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Identification of Antigens of Pathogenic Free-Living Amoebae by Protein Immunoblotting with Rabbit Immune and Human Sera

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Prominent antigens of pathogenic and nonpathogenic free-living amoebae were identified by using polyclonal rabbit immune sera in immunoblot assays. The intent was to determine if prominent epitopes identified with rabbit immune sera could also be recognized by human sera. With rabbit sera, the development of immunoreactive bands was restricted to molecular masses of greater than 18.5 kDa for *Naegleria*, *Hartmannella*, and *Vahlkampfia* antigens. Two or more broad bands of less than 18.5 kDa were prominent features in three different *Acanthamoeba* species. Few cross-reactive antibodies could be detected between representative species of the three different subgroups of *Acanthamoeba*. *Naegleria* antigen was likewise serologically distinct, as were *Hartmannella* and *Vahlkampfia* antigens. The relative lack of cross-reacting antibodies between the pathogenic amoebae suggested that it would be desirable to use a panel of amoebic antigens to represent the range of serologically distinct antigens for assessing reactive antibodies in human sera. In pooled human serum (10 serum specimens per pool), the appearance of minimally reactive bands ranging from 32.5 to 106 kDa was a common feature of all six antigens. A prominent band of less than 18.5 kDa was identified in the *Acanthamoeba culbertsoni* antigen lane in 2 of the 10 human serum specimen pools. When the sera from each of the two groups were tested individually by immunoblotting, the reaction with the *A. culbertsoni* antigen could be associated with one individual. By using a panel of amoebic antigens, this method could prove useful in recognizing undiagnosed amoebic infections by revealing specific reactive antibodies.

The genera *Acanthamoeba* and *Naegleria* include species of free-living soil and water amoebae that are widespread in the environment and that have the potential to produce life-threatening illness. To date, the pathogenic potentials of certain species within these genera have been adeptly demonstrated in laboratory animals and have occasionally been observed in humans (6, 12, 13, 27). In humans, *Naegleria fowleri* amoebic meningoencephalitis is associated with intranasal instillation of amoebae during swimming. The amoebae subsequently penetrate the central nervous system via the olfactory nerves. *Acanthamoeba* species may also produce meningoencephalitis, but the illness in humans may be more chronic, with resulting granuloma formation. Although the A-1 strain of *Acanthamoeba culbertsoni* invades the central nervous systems of experimental animals by first penetrating the nasal mucosa and then spreading to the brain via the olfactory nerves, *Acanthamoeba* species may also spread to the lower respiratory tract and other sites hematogenously. *Acanthamoeba* species can also infect the cornea (*Acanthamoeba* keratitis), and this disease is often associated with contact lens wear (12, 15). Studies with immune sera from laboratory rabbits demonstrated that the species of *Acanthamoeba* share some common antigens, but they can be subdivided into distinct serogroups (5, 16, 24). In addition, species of the genus *Naegleria* share common antigens but also have distinct epitopes, as demonstrated by using the Western blot (immunoblot) methodology (14). *Naegleria* immune serum does not cross-react with *Acanthamoeba* antigen (12).

The purpose of the investigation described here was to identify prominent epitopes of certain pathogenic and nonpathogenic free-living amoebae by using rabbit immune sera and the Western blot methodology. Subsequently, human sera from patients and apparently healthy individuals were screened by immunoblotting for antibodies reactive with amoebic antigens. The majority of the human serum specimens screened were obtained from Army recruits who suffered from acute respiratory disease (ARD). Viral agents such as adenovirus or influenza virus as well as *Mycoplasma pneumoniae* have previously been identified as occasional causative agents of ARD in Army recruits (8, 26). Despite attempts at isolation and serological surveillance programs, the etiologic agent in many cases of ARD has remained unidentified. A logical extension of these studies would be attempts to associate with ARD other potentially infectious agents such as amoebae.

Amoebae have repeatedly been cultivated from the upper respiratory tracts of humans in different surveys. The incidence of amoebae in upper respiratory tract samples has ranged from less than 1% to as high as 24% (1, 4, 11, 25). It has not been clearly determined whether the occurrence of amoebae represents the outgrowth of transient cysts that were inadvertently trapped in the respiratory tract or if the amoebae were in the active motile trophozoite form in vivo. Amoebae have been observed in the lung tissues of laboratory animals which have been experimentally infected intranasally. The possibility exists that amoebae also could cause lower respiratory tract disease in humans.

It was the intention of our study to determine if the

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prominent amoebic epitopes identified by using rabbit immune sera could be identified in human sera by using different amoebic antigens of four different genera. *A. culbertsoni*, *Acanthamoeba polyphaga*, and *Acanthamoeba astronyxis* were each used as sources of antigen. Each species is a representative of a different subgroup of *Acanthamoeba* on the basis of morphology, isoenzyme analysis, and serology (23). Three other genera were represented by using antigens of *N. fowleri* and the nonpathogenic free-living amoebae *Hartmannella vermiformis* and *Vahlkampfia avara*. On the basis of the taxonomic scheme of Page (17), the genus *Hartmannella* and particularly the genus *Vahlkampfia* are considered to be closely related to the genus *Naegleria* (17). The serologic features and antigenic characteristics of these two genera are less well known than are those of the genera *Naegleria* and *Acanthamoeba*.

This report extends the available information regarding the serologic relationships and prominent epitopes associated with the pathogenic free-living amoebae. Certain epitopes representing different amoeba species are occasionally recognized by adult human serum. This documentation of electrophoretically separated antigens and reactive human sera supports the concept that humans are exposed to antibody-inducing levels of antigen from both pathogenic and nonpathogenic amoebae. With additional studies, immunoblots with human sera and amoebic antigens could be useful in recognizing this exposure, the associated pathologic manifestations, and both clinical and subclinical infections with the pathogenic free-living amoebae.

MATERIALS AND METHODS

Amoeba cultivation. Amoebae were cultivated axenically in 75-cm^2 tissue culture flasks containing medium that promoted the optimum growth for each species. *A. culbertsoni* ATCC 30171 and *A. polyphaga* ATCC 30461 were grown in Trypticase soy broth. Plate count broth (Difco, Detroit, Mich.) was used for cultivating *A. astronyxis* ATCC 30137. Medium H4 was used for cultivating *V. avara*, *H. vermiformis*, and *N. fowleri* (2). Cells were incubated at 25°C (except for *N. fowleri* cells, which were incubated at 35°C) and were harvested after a monolayer was formed in approximately 72 h. *H. vermiformis* was kindly provided by Barry Fields from the Centers for Disease Control and Prevention, Atlanta, Ga.; *N. fowleri* Lee was kindly provided by Francine Marciano-Cabral of Virginia Commonwealth University, Richmond.

Antigen and immune serum preparation. Amoebae were harvested by placing the culture flasks on ice to dislodge adherent cells. The amoebae were pelleted by centrifugation (400 × *g*) and were washed three times in sterile phosphate-buffered saline (PBS; pH 7.6). Immunizing antigen was prepared by adjusting the concentration to 2×10^5 cells per ml in PBS and then freezing-thawing four times with liquid nitrogen. New Zealand White rabbits (weight, 2 kg) were immunized once weekly with 2 ml of the antigen preparation for 4 consecutive weeks. Antigen was delivered via the marginal ear vein. One week after the final immunization, the rabbits were bled by cardiac puncture. Washed cells for electrophoresis were prepared for Pierce BCA protein determination (Pierce, Rockford, Ill.) by solubilizing amoeba pellets in 100 µl of 1% sodium dodecyl sulfate (SDS). The soluble protein was boiled for 5 min in a 0.1 M dithiothreitol reducing buffer prior to electrophoresis.

Human sera. The human serum samples used in immunoblots consisted of five banked infant and seven adult serum samples stored at the Indiana University Medical Center. One hundred additional human serum samples drawn from Army recruits diagnosed with ARD were provided by Letterman Army Medical Center, San Francisco, Calif. To facilitate the number tested, serum samples from Army recruits were pooled into 10 groups of 10 sera, with each serum present in its respective pool at a dilution of 1:200. Serum pools were subsequently tested for their reactions with the electrophoresed amoebic antigens.

Electrophoresis and immunoblotting. SDS polyacrylamide gel electrophoresis (PAGE) was performed by using a Mini-Protein II Dual Slab Cell apparatus (Bio-Rad, Hercules, Calif.). Five micrograms of amoebic protein was loaded into each lane of a 1.5-mm 15% acrylamide gel with a 4% stacking gel (preweighed acrylamide-bisacrylamide [37.5:1] mixture; Bio-Rad). Complete electrophoresis occurred in approximately 35 min at 200 V with the voltage held constant. Gels to be stained for protein were stained with Coomassie blue. Proteins were transferred onto 0.45-µm-pore-size nitrocellulose Trans-Blot Transfer Medium (Bio-Rad) by using a Mini Trans Blot Electrophoretic Transfer Cell with a Bio Ice unit (Bio-Rad). Complete transfer occurred at 130 V, while the current ranged from 250 to 350 mA over 60 min.

Following transfer, blots were washed in PBS for 10 min in glass staining dishes on a clinical rotator (Fisher, Pittsburgh, Pa.) at slow speed. They were then blocked for 30 min at room temperature with 1% bovine serum albumin diluted in PBS with 0.05% Tween 20 (pH 7.4, PBS-Tween). After washing the blots twice in PBS-Tween for 5 min each time, they were incubated in the diluted serum at room temperature for 60 min. Preliminary studies comparing the intensities of staining of immunoblots by using a range of dilutions yielded optimum dilutions for rabbit and human sera. From 1:2 serial dilutions of hyperimmune rabbit sera ranging from 1:100 to 1:10,000, a dilution of 1:1,000 was selected. From 1:2 serial dilutions of human sera ranging from 1:10 to 1:1,000, a dilution of 1:200 was selected. Blots were then washed twice in PBS-Tween for 5 min each time before the conjugate was added. The alkaline phosphatase conjugate (Sigma, St. Louis, Mo.) of anti-rabbit immunoglobulin G (IgG, whole molecule) was diluted 1:20,000 in PBS-Tween, or the alkaline phosphatase conjugate (Sigma) of anti-human IgG (gamma chain specific) was diluted 1:2,500 in PBS-Tween. Immunoblots were incubated in the diluted conjugates at room temperature for 30 min. Before adding the substrate, the immunoblots were washed twice for 5 min in PBS-Tween and then once for 5 min in PBS. Immunoblots were then developed in 5-bromo-4-chloro-3-indolylphosphate toludinium–Nitro Blue Tetrazolium Color Development Solution (Bio-Rad) for 10 min at room temperature.

Absorption. For absorption studies, 5×10^5 amoebae (formalin fixed and washed in PBS) were incubated in a 1-ml volume containing 40 µl of human serum and 960 µl of PBS, and the incubation mixture was rocked at 35°C for 1 h. This preparation was then centrifuged (400 × *g*), and the serum supernatant was subsequently diluted to 1:200 before immunoblotting.

Immunofluorescence. For immunofluorescence testing, an indirect fluorescent-antibody procedure described by Sheets et al. (22) was used. The procedure uses formalin-fixed amoebae and incorporates a primary antibody of either hyperimmune rabbit serum or human serum. The secondary antibody is affinity-purified goat anti-rabbit or anti-human fluorescein-conjugated IgG (Sigma).

RESULTS

Whole amoeba lysates subjected to SDS-PAGE were stained with Coomassie blue (Fig. 1A). Multiple protein bands were apparent in each species. Most bands were greater than 18.5

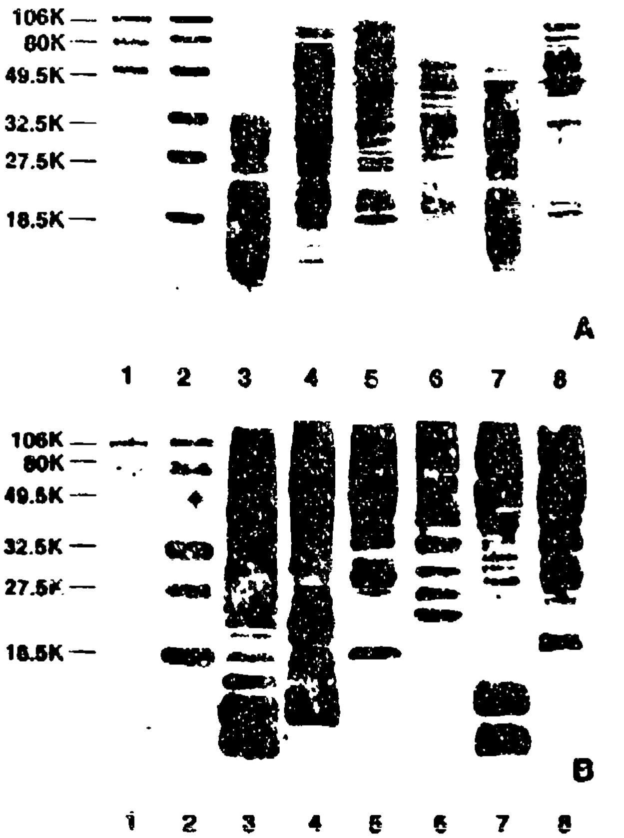

FIG. 1. (A) SDS-PAGE of different amoeba species stained with Coomassie blue. The low- and high-molecular-weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*. (B) Immunoblot panel representing the total number of immunoreactive protein bands of amoeba antigens treated with pooled rabbit immune sera composed of immune serum from each of the six amoeba species (1:1,000 final dilution each). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*.

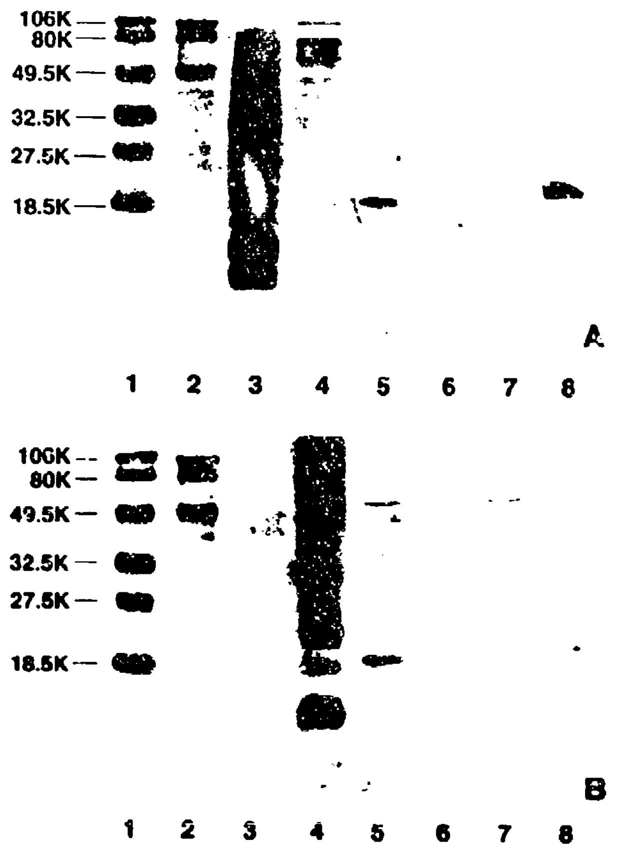

FIG. 2. (A) Immunoblot panel of amoeba antigens treated with only *A. culbertsoni* rabbit immune serum (1:1,000 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*. (B) Immunoblot panel of amoeba antigens treated with only *A. astronyxis* rabbit immune serum (1:1,000 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*.

kDa, with the exception of a broad diffuse area with heavy bands in both *A. polyphaga* and *A. culbertsoni* of less than 18.5 kDa. Each of the three *Acanthamoeba* species had a different banding profile. In *A. astronyxis*, the deeply staining band between 27.5 and 32.5 kDa was unique, and the most numerous and densely staining bands were greater than 27.5 kDa. *A. culbertsoni* showed its most intensely staining bands at less than 27.5 kDa. *A. polyphaga* had prominent bands that ranged from less than 18.5 to 49.5 kDa. *V. avara* and *H. vermiformis* shared a number of minor and major protein bands, with the primary differences related to the intensity of band staining rather than to the presence of different proteins. *N. fowleri* was distinct from the other genera, but its protein profile was likewise composed of many bands which were most numerous at greater than 27.5 kDa.

The total number of immunoreactive protein bands resolved by immunoblotting with the six pooled hyperimmune rabbit serum samples is illustrated in Fig. 1B. The bands represent the epitope reactions of each amoeba species with both homologous and heterologous immune sera. Immunoreactive bands for *Naegleria*, *Hartmannella*, and *Vahlkampfia* antigens developed at molecular masses of greater than 18.5 kDa. In each *Acanthamoeba* species, two or more broad bands of less than 18.5 kDa were prominent features. By Coomassie blue staining, there were relatively low numbers of protein bands in areas above 32.5 kDa in the *A. culbertsoni* lysate, below 18.5 kDa in *A. astronyxis*, and above 49.5 kDa in *A. polyphaga*; however, the Western blot showed several major bands in these areas, indicating that these proteins were highly immunogenic. The presence of multiple bands by Western immunoblotting was a common feature of each species.

The occurrence of cross-reacting antibodies was assessed by allowing the panel of transferred amoebic antigens to react with individual rabbit immune serum (Fig. 2 to 4). Numerous well-defined bands were observed only with the homologous antigen at a rabbit immune serum dilution of 1:1,000 with 5 μg of amoebic antigen per lane. Few cross-reacting antibodies were detected between the three *Acanthamoeba* species chosen to represent different subgroups of the genus (Fig. 2 and 3A). *N. fowleri* was similarly serologically distinct (Fig. 3B). Despite the similarities in polyacrylamide gels stained with Coomassie blue, the immunoblots of *H. vermiformis* and *V. avara* were relatively distinct. *H. vermiformis* antiserum re-

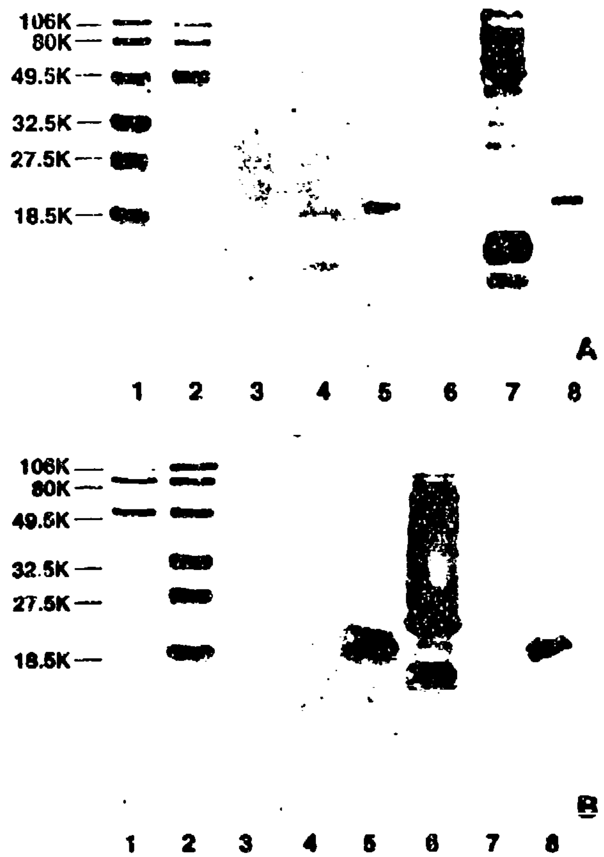

FIG. 3. (A) Immunoblot panel of amoeba antigens treated with only *A. polyphaga* rabbit immune serum (1:1,000 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*. (B) Immunoblot panel of amoeba antigens treated with only *N. fowleri* rabbit immune serum (1:1,000 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*.

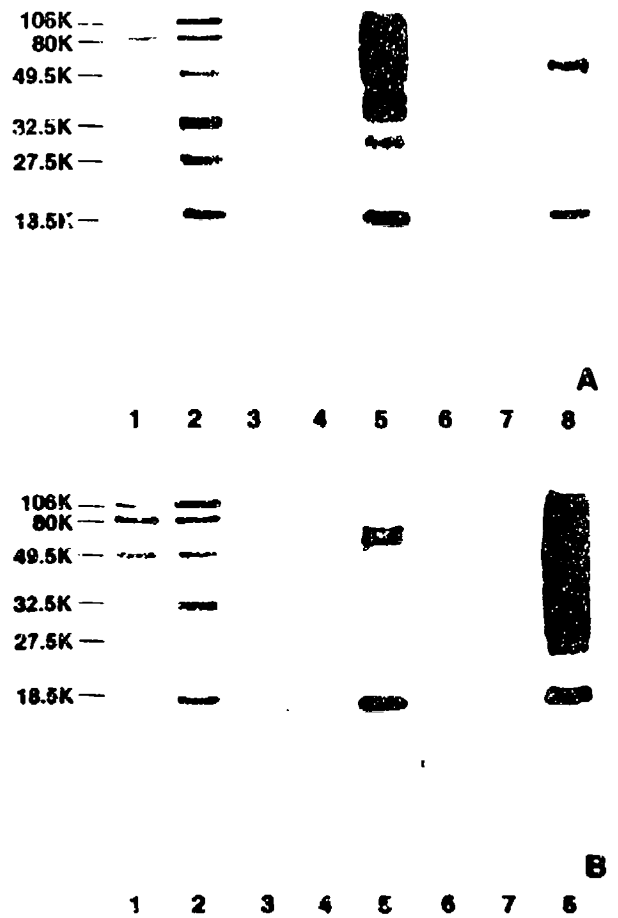

FIG. 4. (A) Immunoblot panel of amoeba antigens treated with only *H. vermiformis* rabbit immune serum (1:1,000 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*. (B) Immunoblot panel of amoeba antigens treated with only *V. avara* rabbit immune serum (1:1,000 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*.

vealed shared bands between 49.5 and 80 kDa (Fig. 4A), and *V. avara* antiserum revealed several shared bands between 32.5 and 106 kDa (Fig. 4B). A prominent band between 14 and 21 kDa was always seen in the *Hartmannella* and the *Vahlkampfia* lanes. This band was also observed in control blots consisting of only the affinity-purified immunoglobulin conjugate of goat anti-human IgG or goat anti-rabbit IgG (no rabbit immune or human serum was used) and appeared to be attributable to the affinity of each conjugate to an unidentified amoebic protein.

Because of the lack of prominent cross-reactive amoebic antigens, a panel of amoebic antigens representing serologically distinct epitopes was used to screen human serum samples for amoeba-reactive antibodies. In 2 of the 10 pools of serum from Army recruits, a prominent band of less than 18.5 kDa was located in the *A. culbertsoni* antigen lane (Fig. 5A). When the sera from each group were tested individually by immunoblotting, the reaction with the *A. culbertsoni* antigen could be associated with one individual from each group. Lightly reactive bands of greater than 32.5 kDa were seen in all 10 serum pools tested (Fig. 5A). Little reactivity was observed in immunoblots of serum from seven laboratory personnel; however, a prominent band of approximately 18.5 kDa was present in the *A. culbertsoni* antigen lane of one individual (Fig. 5B). This band was comparable to the one observed in two of the serum samples from the Army recruit group.

The specificity of human antibodies reactive to *A. culbertsoni* antigen was assessed by absorption with fixed whole cells. A comparison of absorbed and unabsorbed sera showed that the *A. culbertsoni*-reactive antibody was removed by the absorption with homologous antigen (data not shown). Banked infant sera from infants less than 6 months old were also tested by immunoblotting against the panel of amoebic antigens. No evidence of amoeba-reactive antibodies was present in any of the five infant serum samples.

Immunofluorescence testing showed that human serum reactive to *A. culbertsoni* antigen via immunoblotting also caused formalin-fixed *A. culbertsoni* to fluoresce under UV light. Negative controls made with human serum that was unreactive to *A. culbertsoni* antigen did not result in the fluorescence of the formalin-fixed amoebae.

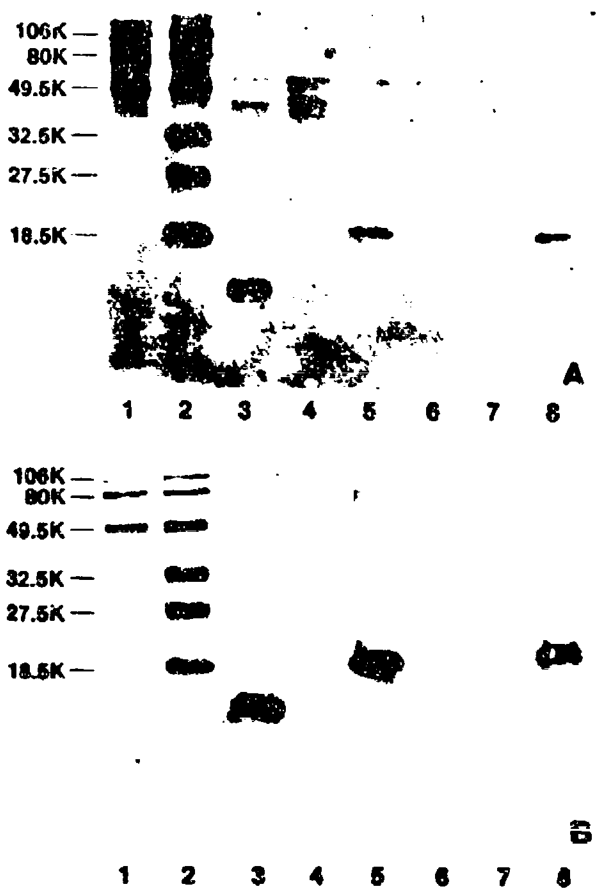

FIG. 5. (A) Immunoblot panel of amoeba antigens treated with 10 pooled serum samples from Army recruits with ARD (1:200 final dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*. (B) Immunoblot panel of amoeba antigens treated with the serum of a laboratory worker (1:200 dilution). Molecular weight markers (lanes 1 and 2) are shown on the left (in thousands). Lanes: 3, *A. culbertsoni*; 4, *A. astronyxis*; 5, *H. vermiformis*; 6, *N. fowleri*; 7, *A. polyphaga*; 8, *V. avara*.

DISCUSSION

Several species of *Acanthamoeba* and one species of *Naegleria* are capable of producing disease in humans (12, 15). The route of infection, the course of the disease, and preventive measures have been defined for certain amoebic diseases such as amoebic meningoencephalitis caused by *Naegleria* species and keratitis caused by *Acanthamoeba* species (9, 12, 13). The factors involved in the spread of pathogenesis of other forms of *Acanthamoeba*-associated human illness are less clear. Although *Acanthamoeba* meningoencephalitis has been most frequently encountered in immunocompromised individuals, the majority of the population undoubtedly has been exposed to these amoebae in the environment. *Legionella pneumophila* can use *Naegleria* and *Acanthamoeba* organisms as host cells for intracellular replication, and Rowbotham (20, 21) hypothesized that amoebae could also serve as vectors for the delivery of *Legionella* bacteria to humans.

To better understand the environmental transmission of amoebae to humans and the epidemiology of disease involving free-living amoebae, various serologic methods have been used to assess the presence of amoeba-reactive antibody in humans. The serologic methods used previously have included the indirect fluorescent-antibody assay, complement fixation, agglutination, indirect hemagglutination, and Western blot analysis (3, 7, 10, 14, 19). Results of those studies suggest that humans have naturally occurring amoeba-reactive antibodies represented by IgG and IgM. At the present time, it is not known if these antibodies are a reflection of environmental exposure to amoebic antigen, the consequence of subclinical infection, or the result of cross-reacting antibodies from some other source.

The number and character of *Naegleria* epitopes identified by using human serum have been examined by Marciano-Cabral et al. (14) by Western blot analysis. Their results suggested that human IgG reacts primarily with internal amoebic antigens, and these antigens show extensive cross-reactivity among species. Conversely, the amoeba-agglutinating activity of normal human serum is a function of IgM reactivity with surface antigens. These antibodies are species specific. Antibodies from human serum react to both pathogenic and nonpathogenic *Naegleria* species, and individuals appear to have been exposed to different amoebic antigens. In a study by Dubray et al. (7), immunoblot analysis of selected human serum against *N. fowleri* or *Naegleria lovaniensis* antigen demonstrated four bands of reactivity. The results suggested that human antibodies recognized identical antigenic sites in both of these species.

Most recently, Moura et al. (16) compared the antigenic characteristics of 13 *Acanthamoeba* strains by immunoblot analysis by using homologous and heterologous rabbit immune sera. Most bands were observed between 20 and 116.5 kDa, and the staining intensity was often so great that it was difficult to distinguish individual bands. In some instances, antibodies cross-reacted extensively among the 13 strains. Certain antigens were more extensively shared within each of three *Acanthamoeba* morphologic groupings on the basis of immunoblot profiles. It was also apparent that certain subgroups of *Acanthamoeba* were more closely related serologically.

Results from the present study demonstrate the complex protein profiles associated with the free-living amoebae following SDS-PAGE. It is apparent that the majority of these proteins are highly immunogenic in laboratory rabbits. A notable observation is the relative lack of cross-reacting antibodies among the selected amoebae by immunoblotting. Even within the genus *Acanthamoeba*, the selected type species from each subgroup were serologically distinct. These differences are consistent with the division of *Acanthamoeba* species into distinct subgroups, in part on the basis of serology. A prominent feature of our study with *Acanthamoeba* species was the observation of distinct broadly immunoreactive proteins of less than 18.5 kDa. These bands were observed only in immunoblots of the three *Acanthamoeba* species with homologous rabbit immune serum and in the *A. culbertsoni* antigen lanes of approximately 3% (3 of 112) of the immunoblots developed with human serum. Immunofluorescence testing confirmed the reactivity of one of these three human serum samples. The supply of the other two serum samples was exhausted during immunoblotting and absorption assays.

No distinction has been made between the two reactive serum samples from Army recruits and normal adult serum taken at Indiana University. It is likely that additional studies representing other groups of humans will be needed to define the range of naturally occurring amoeba-reactive antibodies in humans. Previously, investigators have demonstrated by immunoblotting that certain individuals have naturally occurring antibodies that react with protozoa such as *Toxoplasma gondii*

(18). The source of the antigenic stimulation was unknown. Studies have suggested antigenic similarities between *T. gondii* and other organisms that are not considered to be pathogens and that rarely occur in humans (18). The specificity of amoeba-reactive antibodies detected with our assay was confirmed by absorption studies with *A. culbertsoni* antigen. However, the exact events associated with antigen exposure or antibody induction in our investigation cannot be determined. Our observations support the concept that previous exposure to *A. culbertsoni* may be responsible for the occurrence of reactive antibodies in this small group of individuals. The ability of the immunoblot to detect human antibodies that specifically react with *A. culbertsoni* epitopes suggests a likelihood of detecting patient seroconversions to this amoeba and other serologically distinct species of *Acanthamoeba* as well.

Human antibodies reactive with *N. fowleri* antigen were detected in pooled serum from Army recruits. In one blot, a sharp doublet was observed at 49.5 kDa (data not shown). Other antibodies reactive with *N. fowleri* were detected by the presence of three to four bands between 32.5 and 106 kDa. The appearance of these bands did not differ from the appearance of similar bands of the same molecular size in the lanes of the other five amoebic antigens. These bands were not as prominent or deeply staining as those reported by other investigators who used immunoblotting to assess the reactivity of human serum with *N. fowleri* antigen (7, 14). This could be, in part, a reflection of the test parameters such as antigen concentration, serum concentration, or other variables. Other investigators used a 1:50 human serum dilution, whereas a 1:200 serum dilution was used in the present study. In our study, we did not note major differences in blot results when the serum dilution was reduced from 1:200 to 1:50. This lower dilution resulted mainly in increased background staining. Our investigation also represents a study population different from that of other investigators, and as suggested previously, differences in results with the *Naegleria* antigen used in immunoblotting may represent geographical variation in amoebic antigen exposure (14).

The similarity between *H. vermiformis* and *V. avara* in the Coomassie blue-stained gel was pronounced. These two genera are closely related morphologically, and the similarities in protein bands would serve to underscore this similarity. Additional gel electrophoresis of these two strains was performed on replicate cultures from the American Type Culture Collection, and the nearly identical Coomassie blue staining patterns were again apparent. To our knowledge, these two strains of amoebae have not previously been compared by SDS-PAGE. The results of this initial comparison indicate that additional studies are needed to determine the significance of this similarity and if this is a consistent feature of other strains and species in these two genera. These results demonstrate that these two species from different genera are remarkably similar by SDS-PAGE, yet they can be distinguished by immunoblotting. The consistent appearance of nearly identical bands between 14 and 21 kDa in only the *Hartmannella* and *Vahlkampfia* antigen lanes also merits additional study. The visualization of these bands in the absence of rabbit immune or human serum suggests that the electrophoresed proteins directly bind some component of the alkaline phosphatase-immunoglobulin conjugate. The ability of certain components of microorganisms to bind immunoglobulins in a nonimmune fashion, as observed with protein A, has been well established. This has not been observed previously in the pathogenic free-living amoebae.

This report documented the major antibody-reactive antigens in six species of free-living amoeba. Reactions with rabbit immune sera demonstrated that each amoeba was serologically distinct, and there was little cross-reacting antibody. This insight has important implications for future attempts to diagnose human amoebic infections serologically. Apparently, it will be necessary to use a battery of amoebic antigens representing the serologically distinct species and genera in order to recognize the presence of amoeba reactive antibodies. This study provides baseline information on the presence of antibodies reactive with free-living amoebae in human serum and suggests the feasibility of using immunoblotting to detect seroconversions in individuals infected with the pathogenic free-living amoebae.

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